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Maternally Expressed Gene with Localized mRNA Encodes a Protein with *Caenorhabditis elegans* MEX-3-like KH Domains[1]

Yutaka Satou

Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Maternal factors localized in the posterior-vegetal cytoplasm of an ascidian egg are essential for cell specification and pattern formation of the embryo. The molecular identification of the localized factors and the elucidation of the machinery associated with the localization are therefore key research subjects. I report here the isolation and characterization of a novel maternally expressed gene, *posterior end mark 3* (*pem-3*). The *pem-3* cDNA was obtained from a cDNA library of fertilized egg mRNAs subtracted with gastrula mRNAs of *Ciona savignyi*. As in the case of *pem* (Yoshida *et al.*, 1996, *Development* 122, 2005–2012), the *pem-3* maternal transcript was gradually concentrated after fertilization in the posterior-vegetal cytoplasm of the egg, and it later marked the posterior end of developing embryos. The PEM-3 protein was also detected in the posterior end of early embryos. The nucleotide sequence predicted that *pem-3* encodes a probable RNA-binding protein with two KH domains that have an extensive similarity with those of *Caenorhabditis elegans* MEX-3. MEX-3 is also localized in nematode embryos (Draper *et al.*, 1996, *Cell* 87, 205–216), suggesting that PEM-3 is a candidate homologue of MEX-3. In addition to maternal expression, a zygotic transcript of *pem-3* and its gene product were detected in cells of the neural plate, mesenchyme, and epidermis of embryos after the neural-plate stage. Inhibition of zygotic expression using an antisense oligonucleotide resulted in the development of abnormal larvae without sensory pigment cells, suggesting that the zygotic PEM-3 plays a role in the differentiation of the brain of the ascidian larva.

Key Words: **Ascidian; localized maternal mRNA; *pem-3;* KH domain; RNA-binding protein.**

INTRODUCTION

Using ascidian eggs, Chabry (1887) described the first blastomere destruction experiment in the history of embryology. He found that if the cells of early ascidian embryos are destroyed, they cannot be replaced or compensated for by other cells during development. Since then, the ascidian egg has been regarded as a "mosaic" egg, in which several types of embryonic cells are specified autonomously dependent on prelocalized egg cytoplasmic factors or determinants (reviewed by Satoh, 1994). Recent experimental embryological studies have provided convincing evidence of the localization of the determinants responsible for the differentiation of muscle (Nishida, 1992; Marikawa *et al.*, 1994), epidermis (Nishida, 1994a), and endoderm (Nishida, 1993), factors for the establishment of the embryonic anteroposterior axis (Nishida, 1994b), and those for the initiation of gastrulation (Jeffery, 1990; Nishida, 1996). The molecular identification of localized maternal factors, the elucidation of the machinery responsible for the localization, and the exploration of the mode of action of the localized factors are therefore key research subjects for the elucidation of the pattern formation of ascidian embryos (cf. Satoh *et al.*, 1996).

Various molecular approaches have revealed some developmentally important maternal factors in ascidian eggs (e.g., Swalla and Jeffery, 1995, 1996). In a previous study, Yoshida *et al.* (1996) isolated and characterized a novel maternal gene in the ascidian *Ciona savignyi*. The gene was named *posterior end mark* (*pem*), because the transcript is

[1] Nucleotide sequence data reported in this article have been deposited with the DDBJ, EMBL, and GenBank Data Libraries under Accession No. AB001770.

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initially concentrated in the posterior-vegetal cytoplasm of the fertilized egg, and later the distribution of the transcript marks the posterior end of developing embryos. Although the predicted amino acid sequence of the PEM protein showed no significant similarity to known proteins, the overexpression of this gene produced by a microinjection of synthetic mRNA into fertilized eggs resulted in the development of tadpole larvae with deficiencies of the anterior-most adhesive organ, dorsal brain, and sensory pigment cells (Yoshida *et al.*, 1996).

During ascidian development, the expression of tissue-specific genes commences much earlier than that of other chordates, reflecting the very early fate determination and subsequent specification of embryonic cells (cf. Satoh, 1994). We have therefore attempted to isolate maternally expressed genes by the subtractive hybridization of mRNAs of fertilized eggs with those of gastrulae. Taking advantage of the well-known lineage and segregation pattern of developmental fates as well as the *in situ* hybridization of whole-mount specimens, we have isolated several cDNA clones of genes with localized mRNAs (*pem-2*, *pem-4*, *pem-5*, and *pem-6;* Satou and Satoh, 1997). Here the gene *posterior end mark 3* (*pem-3*) is reported, so named because, as in the case of *pem* (Yoshida *et al.*, 1996), the distribution of the maternal transcript marks the posterior end of developing ascidian embryos. The PEM-3 protein was also localized in the posterior end of early embryos. The predicted amino acid sequence suggested that *pem-3* encodes a probable RNA-binding protein with two KH domains, which showed an extensive similarity with those of *Caenorhabditis elegans* MEX-3. MEX-3 is localized in anterior blastomeres of early nematode embryos and is also a component of P-granules (Draper *et al.*, 1996). The sequence similarity and localization of maternal PEM-3 and MEX-3 suggest their orthology, even though further investigations are required. In addition to the maternal expression, the zygotic transcript of *pem-3* and its gene product were detected in several regions including cells of the neural plate of neural-plate stage embryos. Inhibition of the zygotic transcripts by the treatment of embryos with an antisense oligonucleotide resulted in the development of abnormal larvae without sensory pigment cells, suggesting that PEM-3 protein produced in the neural-plate cells has an essential role in the differentiation of the brain of the ascidian embryo.

MATERIALS AND METHODS

Ascidian Eggs and Embryos

Ci savignyi adults were collected near the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Iwate, Japan, and maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

RNA Isolation and Northern Analysis

Total RNA was isolated from the fertilized eggs or gastrulae by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan, Tokyo, Japan).

Poly(A)$^+$ RNA was isolated as described above, fractionated by agarose gel electrophoresis, and transferred to a Hybond-N$^+$ membrane (Amersham, Buckinghamshire, UK). Blots were hybridized with a ^{32}P-random-labeled *pem-3* DNA probe in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, and 50% formamide. The filter was washed twice in 2× SSC/0.1% SDS and twice in 0.2× SSC/0.1% SDS at 65°C and exposed to X-ray film.

Screening of the Subtracted cDNA Library

A cDNA library of fertilized-egg mRNA subtracted with gastrula RNA was constructed in our previous study and screened in the same way as described (Satou and Satoh, 1997). Briefly, from the library, clones were randomly picked up and partially sequenced from the poly(A) tail to avoid analyzing the same clones any further. After partial sequencing, the localization of corresponding mRNA was examined for each clone by whole-mount *in situ* hybridization.

Sequencing

Nucleotide sequences were determined for both strands with a dye primer cycle sequencing FS ready reaction kit and ABI Prism 377 DNA sequencer (Perkin–Elmer, Norwalk, CT).

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization was performed basically as described previously (Yasuo and Satoh, 1994) with some modifications. Embryos were fixed in 4% paraformaldehyde in 0.1 M MOPS (pH 7.5), 0.5 M NaCl at 4°C for 16 h, prior to storage in 80% ethanol at −20°C. After thorough washes with PBST (phosphate-buffered saline containing 0.1% Tween 20), the fixed specimens were partially digested with 2 μg/ml proteinase K in PBST for 30 min at 37°C. After being washed with PBST, the specimens were postfixed with 4% paraformaldehyde in PBST for 1 h at room temperature and then washed again with PBST. After soaking in 0.1 M triethanolamine (pH 8.0), the specimens were acetylated with 1% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min and washed again with PBST. After prehybridization at 42°C for 1 h, the specimens were hybridized with DIG-labeled probes at 42°C for 16 h. RNA probes were prepared with a digoxygenin (DIG) RNA labeling kit (Boehringer Mannheim, Heidelberg, Germany). The hybridization buffer contained 50% formamide, 5× SSC, 5× Denhardt's solution, 100 μg/ml yeast tRNA, 0.1% Tween 20, and 0.5 μg/ml DIG-labeled RNA probe.

After hybridization, the specimens were washed in 50% formamide, 2× SSC, 0.1% Tween 20 at 50°C twice and then in RNase buffer [0.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 0.1% Tween 20] three times. The specimens were treated with 20 μg/ml RNase A for 30 min at 37°C, washed in RNase buffer once, washed in 50% formamide, 2× SSC, and 0.1% Tween 20 at 50°C once, and washed in 50% formamide, 0.5× SSC, and 0.1% Tween 20 at 50°C twice. After thorough replacement with PBST, the specimens were blocked with 0.5% blocking reagent (Boehringer Mannheim) in

PBST for 30 min before overnight incubation with 1:2000 alkaline-phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) at 4°C. The specimens were washed in PBST for 30 min four times with rocking and then in alkaline phosphatase buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris–HCl, pH 9.5) twice. For signal detection, the embryos were incubated with NBT/BCIP/alkaline phosphatase buffer following the supplier's instructions (Boehringer Mannheim). The reaction was stopped in PBST. When the signal was weak, the embryos were dehydrated in a graded series of ethanol and then cleared in a 1:2 mixture of benzyl alcohol:benzyl benzoate (BABB).

Injection of Synthetic Capped mRNA

Synthetic capped mRNA for *pem-3* was synthesized from *pem-3* cDNA cloned into pBluescript RN3 vector (Lemaire *et al.,* 1995) using a Megascript kit (Ambion, Austin, TX). To obtain a capped mRNA, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at a final concentration of 6 mM. As a negative control, *lacZ* mRNA was synthesized from *lacZ* gene cloned into pBluescript RN3 vector in the same way. The injection of mRNAs into the fertilized eggs was performed as described previously (Marikawa *et al.,* 1995).

Antisense Oligonucleotides

The following six phosphorothioated oligodeoxynucleotides (ODNs) were custom-synthesized and high-performance liquid chromatography (HPLC)-purified by Greiner Japan (Tokyo). The prepared seven ODNs were AS (antisense)1 (5′-TGCATCATC-TCGTGCCTCATTT-3′), AS2 (5′-CAGGTTGGATAGTTCAAG-3′), AS3 (5′-GCAGCGGATTGAACCTCTCG-3′), SC (scramble)1 (5′-TTAGCCTTAGCCTTAGCCTTCT-3′), SC2 (5′-GACTAGT-TAGGTATCAGG-3′), SC3 (5′-GGACCTCTGAGCTAGAGCC-T-3′), and SN (sense)3 (5′-CGAGAGGTTCAATCCGCTGC-3′). AS1, AS2, and AS3 were antisense ODNs, which correspond to nucleotides 56 to 35, 138 to 121, and 413 to 394, respectively. SC1, SC2, and SC3 were control ODNs that had the same base composition as that of AS1, AS2, and AS3, respectively. SN3 has a sequence from 413 to 394, another control ODN for AS3. Fertilized eggs or later stage embryos were treated with an appropriate concentration of ODN and incubated until hatching. The embryos were cultured in a 24-well plate, and each well contained 20 to 40 embryos in 2 ml seawater. The division of treated embryos was arrested at the 8-, 64-, and 110-cell stages by immersing the embryos in seawater containing 2.5 μg/ml cytochalasin B (Sigma, St. Louis, MO) and ODNs.

Histochemical Staining for Alkaline Phosphatase and Immunostaining for Myosin Heavy Chain

The differentiation of endoderm cells in experimental embryos was monitored by the histochemical reaction of alkaline phosphatase as described by Marikawa and Satoh (1996). Muscle cell differentiation was monitored by immunostaining with Mu-2 antibody as described by Nishikata *et al.* (1987).

Antibody

Two rabbits were immunized with full-length PEM-3 protein expressed in *Escherichia coli.* Antiserum from the two rabbits recognized a band at around 56 kDa, which was comparable to the predicted molecular mass of PEM-3. One of the sera was affinity-purified with the antigen and used for the following analysis.

For Western blotting, samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBST. After incubation with the anti-PEM-3 polyclonal antibody and then horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Amersham), the membrane was incubated with an enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham) and exposed to X-ray film. After signal detection, the membrane was stained with 0.1% amide black in 40% methanol and 10% acetic acid solution for a loading control.

The immunohistochemical staining of embryos was done basically following the protocol described in Takamura (1998) with minor modifications. Embryos were fixed with 4% paraformaldehyde in 0.1 M MOPS (pH 7.5), 0.5 M NaCl at 4°C for 16 h, prior to storage in 80% ethanol at −20°C. The embryos were replaced in PBST and then blocked with 5% fetal bovine serum (FBS) in PBST for several hours. After incubation with 1:50 diluted anti-PEM-3 antibody at 4°C overnight, the embryos were washed with PBST all day. The samples were incubated with 1:800 diluted HRP-conjugated anti-rabbit antibody at 4°C overnight. After being washed as before, they were reacted with DAB substrate (0.06% w/v diaminobenzidine, 0.015% v/v hydrogen peroxide in PBST). After clearing with BABB, they were observed with a light microscope.

RESULTS

pem-3 Encodes a KH Domain Protein

The *pem-3* cDNA was isolated with *pem-2, pem-4, pem-5,* and *pem-6* in our previous study (Satou and Satoh, 1997). The cDNA sequence of *pem-3* was found to be 1980 bp long (Fig. 1). The result of the Northern blotting indicated that the length of the transcripts was about 2.1 kb (Fig. 2). However, the clone might contain the entire coding and 3′ untranslated region (UTR) sequences of *pem-3,* since the 5′ end of the cDNA contained stop codons in all three reading frames in front of the initiator methionine at nucleotide position 37. The cDNA had a single open reading frame of 465 amino acids (Fig. 1). The predicted PEM-3 protein contained 66- and 65-amino-acid regions that were 42% identical to each other (underlined in Fig. 1). Database searches indicated that these repeated sequences correspond to a previously identified protein motif called a KH domain. The KH domain was first described in the hnRNP K protein, a pre-mRNA-binding protein (Siomi *et al.,* 1993). *Ca. elegans* MEX-3 was revealed to be a KH domain protein that regulates blastomere identity in early embryos (Draper *et al.,* 1996).

As shown in Fig. 3A, when compared to other proteins, the KH domains of PEM-3 showed an extensive similarity with those of *Ca. elegans* MEX-3. Within the KH domains, the amino acid sequence of the PEM-3 was 83% identical to that of MEX-3, suggesting that *pem-3* was a candidate

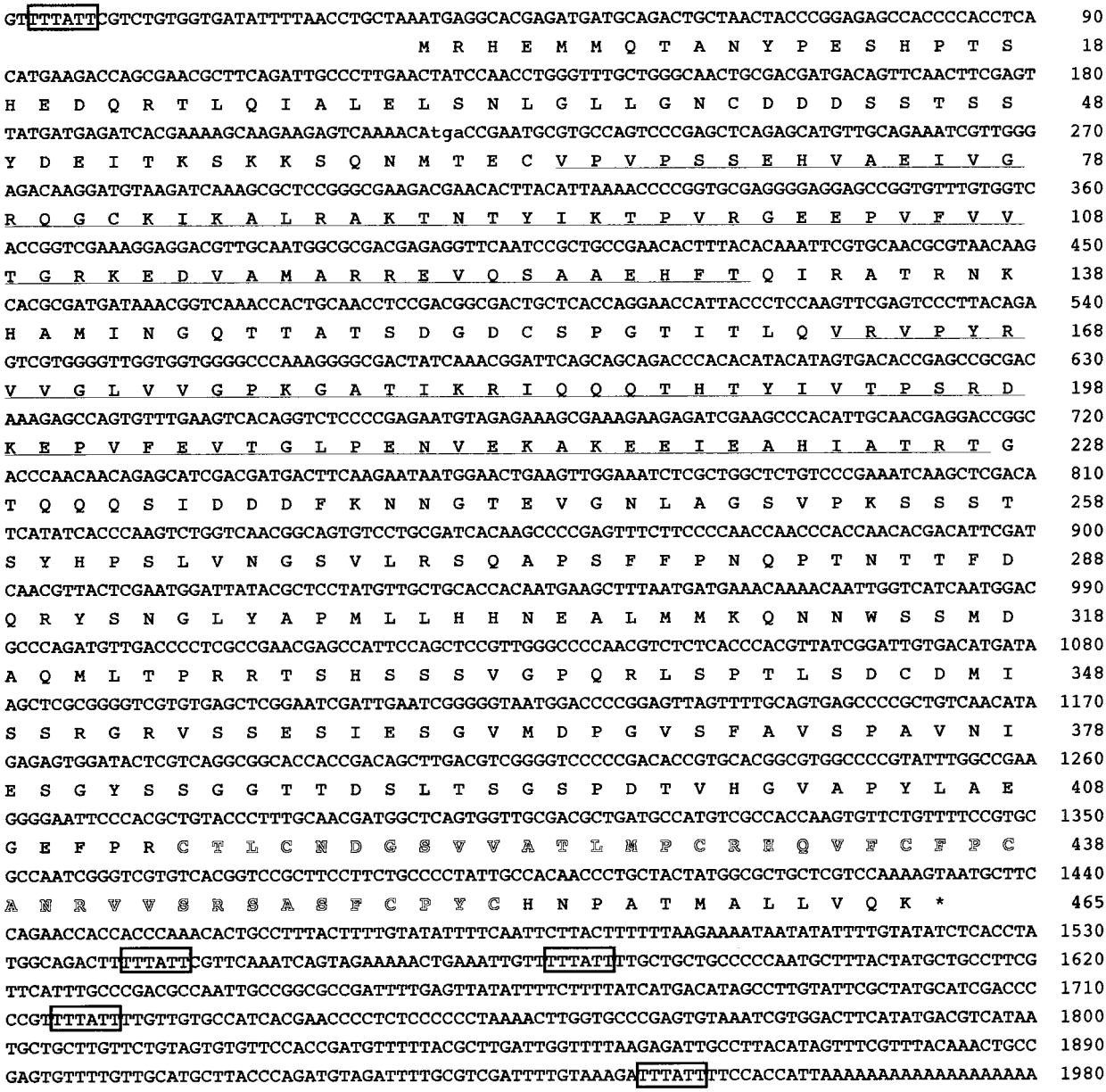

FIG. 1. Nucleotide and deduced amino acid sequence of *pem-3* cDNA clone. The 1980-bp insert includes a single open reading frame that encodes a polypeptide of 465 amino acids. The termination codon is shown by an asterisk. Two predicted KH domains (amino acid positions 65–130 and 163–227) are underlined. The predicted RING finger motif is shown by open letters (amino acid positions 414–453). The 6-bp sequence (TTTATT) in the untranslated region was boxed (see text).

homologue of *Ca. elegans mex-3*. However, the PEM-3 sequence other than the KH domains did not resemble that of MEX-3.

In addition to the KH domains, database searches indicated that PEM-3 also contained the consensus sequence of the RING finger or C3HC4 zinc finger motif (open-letter residues in Fig. 1), which was not found in the sequence of MEX-3. The RING finger motif is represented as CX(I/L/V)$CX_{9\text{-}27}$CXHX(F/L/I)CXXCL/I/$MX_{10\text{-}18}$CPXC (Fig. 3B). It is thought that the RING finger motif is involved in protein–protein interactions (Rothe *et al.*, 1994; Borden *et al.*, 1995; Saurin *et al.*, 1996). The study of the *Ca. elegans* genome showed that RING finger is one of the most common zinc fingers (Clarke and Berg, 1998).

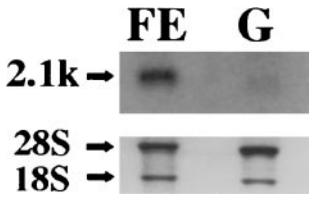

FIG. 2. Isolation of a cDNA clone for a maternal gene, *posterior end mark 3* (*pem-3*), of the ascidian *Ciona savignyi*. Northern blots of poly(A)$^+$ RNA of fertilized eggs (FE) and gastrulae (G) showing a predominance of *pem-3* mRNA in fertilized eggs. Each lane was loaded with 1.8 μg of poly(A)$^+$ RNA, and the amount of loaded RNA is shown by the ethidium bromide staining of 28S and 18S rRNA (bottom).

The Expression Pattern of pem-3 mRNA and PEM-3 Protein

Similar to *pem*, the localization and segregation of *pem-3* mRNA marked the posterior end of developing embryos (Fig. 4). The *pem* transcript appears to be an abundant maternal message, and therefore the dynamic change in its localization and segregation was very clearly revealed by whole-mount *in situ* hybridization from unfertilized eggs to the midtailbud stage (cf. Fig. 2 of Yoshida *et al.*, 1996). However, presumably reflecting a lesser amount of *pem-3* transcript, its distribution pattern was not so evident until the 8-cell stage and was much narrower at later stages than that of *pem*.

In the unfertilized eggs, the hybridization signal appeared in the peripheral cytoplasm, but its localization was not so conspicuous. In ascidian eggs, fertilization evokes a dynamic rearrangement of the egg cytoplasm called ooplasmic segregation, yielding the establishment of the dorsoventral and anteroposterior axes of the embryo (e.g., Sardet *et al.*, 1989). After the first phase of ooplasmic segregation, the *pem-3* hybridization signal became stronger near the vegetal pole but again was not so distinctly localized (data not shown), and after the second phase of ooplasmic segregation, the *pem-3* transcript moved to the subequatorial region to form a rather broad crescent-like structure (Fig. 4A).

The cleavage of ascidian eggs is bilaterally symmetrical. During the first two cleavages, the *pem-3* transcript was distributed over the posterior-vegetal cytoplasmic region of the embryos, but not so much restricted to the cortical cytoplasm (data not shown). At the 8-cell stage, however,

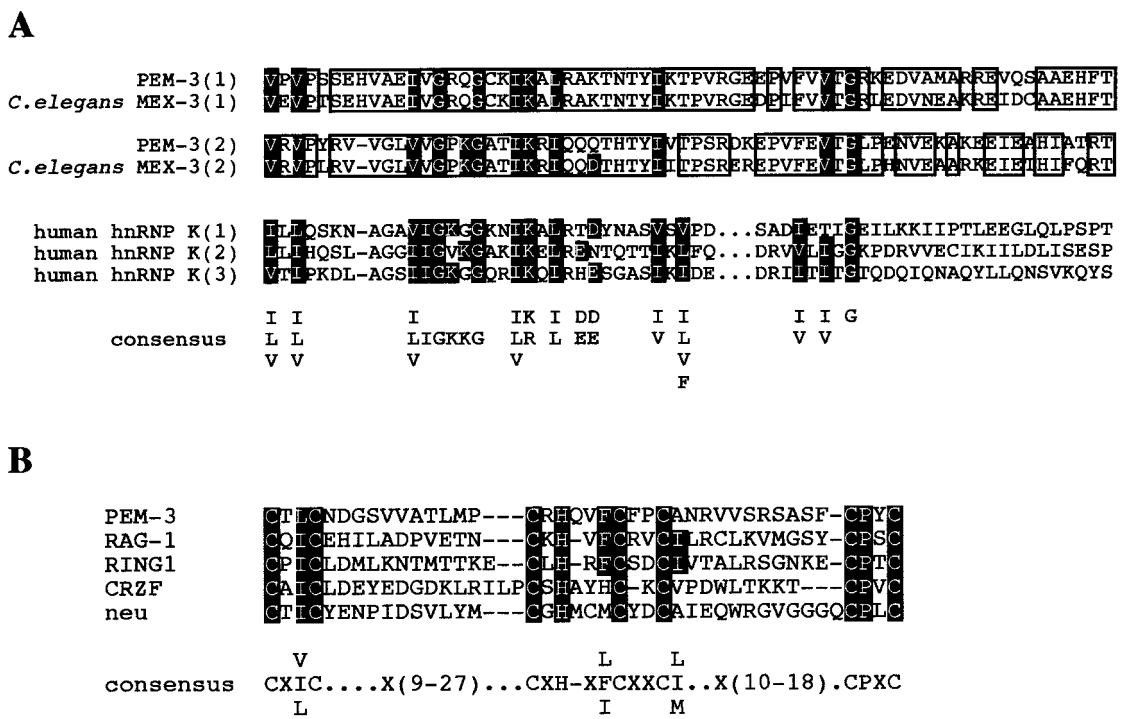

FIG. 3. (A) KH domain sequences of PEM-3, *Ca. elegans* MEX-3 (Draper *et al.*, 1996), and hnRNP K (Siomi *et al.*, 1993). The highly conserved amino acids in all KH domain proteins are boxed in black (adapted from Siomi *et al.*, 1993), and the residues that are conserved between PEM-3 and MEX-3 are enclosed by boxes. (B) The proposed structure of a RING finger in the PEM-3 protein was compared with RAG-1 (Schatz *et al.*, 1989), RING1 (Lovering *et al.*, 1993), CRZF (Tranque *et al.*, 1996), and neu (Price *et al.*, 1993). Consensus sequences were obtained from Freemont *et al.* (1991), Price *et al.* (1993), and Tranque *et al.* (1996).

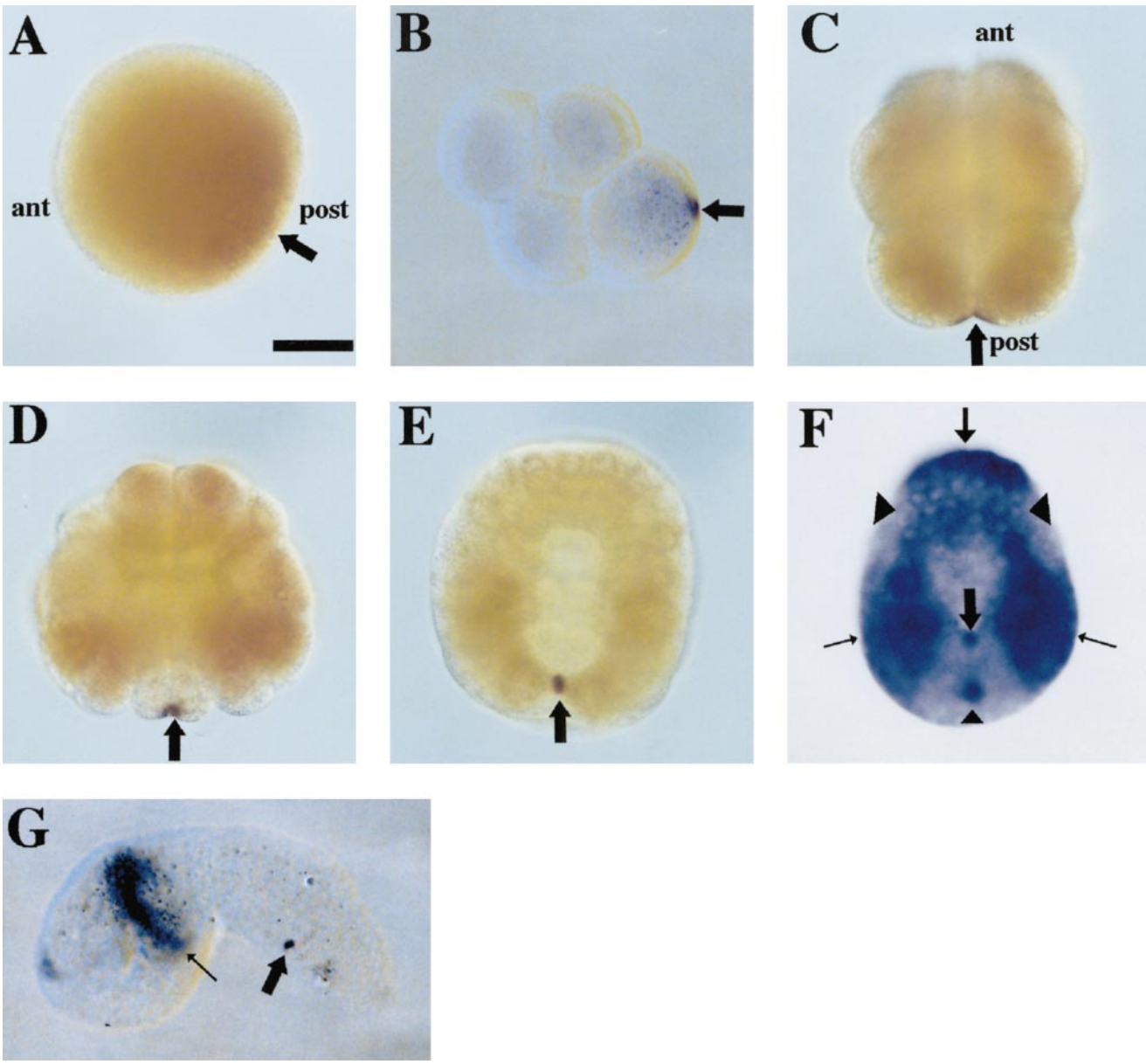

FIG. 4. Distribution of *pem-3* maternal mRNA marks the posterior end of developing embryos, as revealed by whole-mount *in situ* hybridization. (A) A fertilized egg after completion of the second phase of ooplasmic segregation, lateral view. *pem-3* maternal mRNA is seen to be concentrated on the posterior side of the egg (arrow). ant, anterior; post, posterior side of the egg. (B, C) An 8-cell embryo; (B) lateral view and (C) vegetal pole view. Very narrow localization of *pem-3* mRNA is shown by arrows. (D) A 32-cell embryo, vegetal pole view. (E) A midgastrula, vegetal pole view. (F) A neural-plate stage embryo. Zygotic expression is seen in neural plate (large arrowheads), mesenchyme (small arrows), anterior epidermis (middle arrow), and posterior unidentified cells (small arrowhead) in addition to the maternal message (large arrow). (G) A tailbud embryo, lateral view. Zygotic *pem-3* mRNA is detected in mesenchyme (small arrow), in addition to maternal mRNA in an endodermal strand (large arrow). The embryos were photographed in PBST except for B, F, and G. The embryos in B, F, and G were cleared in BABB. Scale bar represents 50 μm for all photographs.

the distribution of the *pem-3* transcript was restricted to the very narrow, posterior region of B4.1 cells (a pair of posterior vegetal blastomeres in the bilaterally symmetrical embryo; Figs. 4B and 4C). At the 16-cell stage, *pem-3* mRNA was found in the posterior-most region of the embryo, only in the posterior cytoplasm of the B5.2 cells (data not shown). Positive hybridization signals for the *pem-3* transcript were detected in the posterior cytoplasm

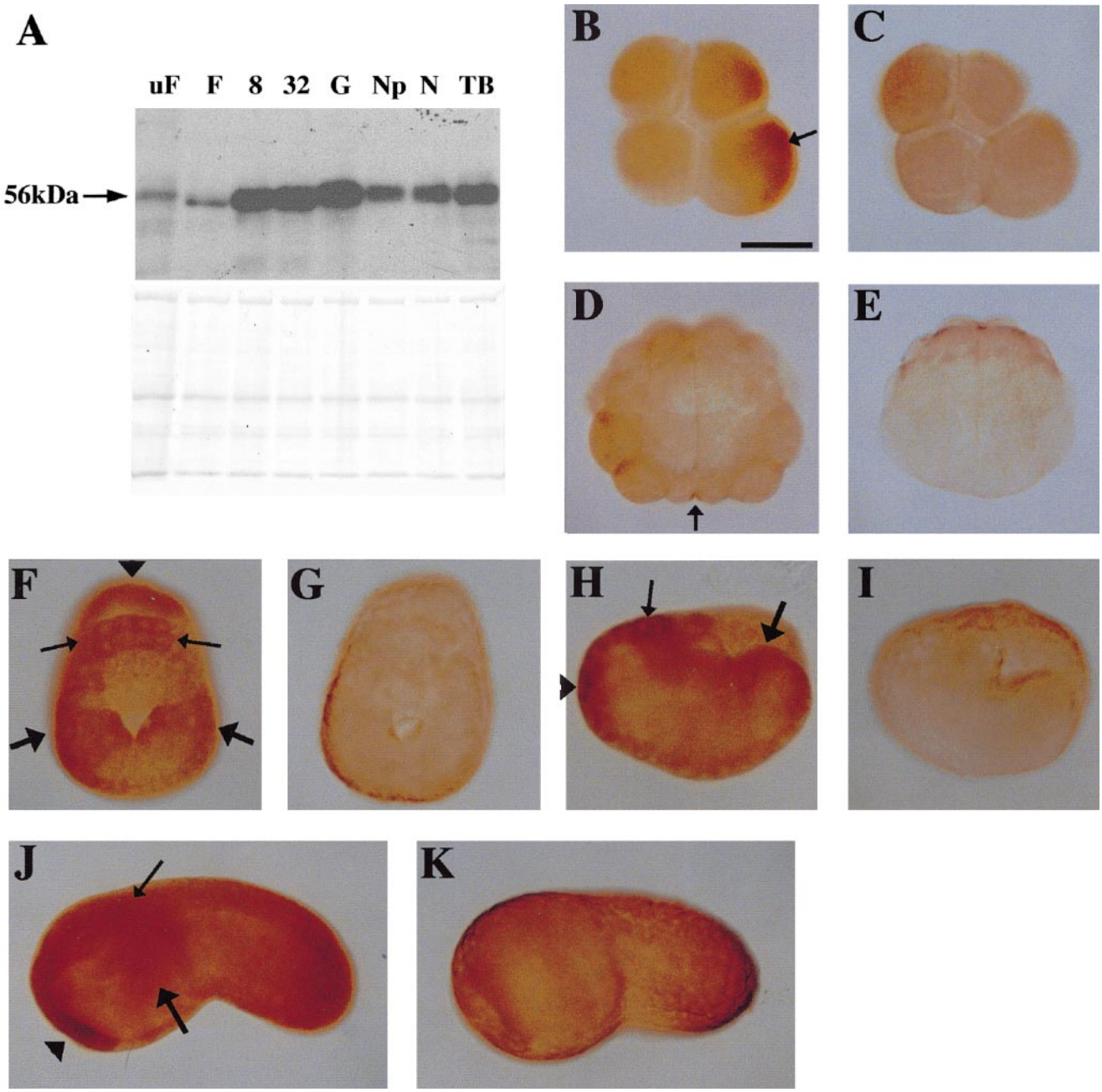

FIG. 5. Expression pattern of PEM-3. (A) Western blotting showing the temporal expression pattern of PEM-3 protein. uF, unfertilized eggs; F, fertilized eggs; 8, 8-cell stage embryos; 32, 32-cell stage embryos; G, gastrulae; Np, neural-plate embryos; N, neurulae; TB, tailbud embryos. Each lane was loaded with the protein of 25 eggs or embryos. Arrow shows the band of PEM-3 protein. (Bottom) A photograph of the membrane stained with amide black after ECL detection for the control showing the amount of loaded protein. (B–K) Whole-mount immunostaining with polyclonal antibody against PEM-3 (B, D, F, H, J) or preimmune antiserum (C, E, G, I, K). (B, C) An 8-cell embryo, lateral view. (D, E) A 32-cell embryo, vegetal pole view. (F, G) A neural-plate stage embryo, dorsal view. (H, I) A neural-plate stage embryo, lateral view. (J, K) A tailbud embryo, lateral view. (B, D) Arrows show very narrow expression of PEM-3 from the maternal message. (F, H, J) PEM-3 from the zygotic message is detected in mesenchyme (large arrows), anterior epidermis (arrowheads), and posterior two rows of neural-plate cells of the neural-plate stage embryo or brain of the tailbud embryo (small arrows). Scale bar represents 50 μm for all photographs.

of B6.3 in the 32-cell-stage embryo (Fig. 4D) and then in B7.6 of the 64-cell-stage embryo (data not shown). At the gastrula stage, the *pem-3* transcript was seen in two invaginating posterior cells (B7.6 blastomeres; Fig. 4E). The *pem-3* signals in embryos up to the gastrula stage appeared to be restricted to a region more posterior than that of the *pem* signals. At the neural-plate stage, the transcript was also seen (Fig. 4F). This maternal transcript was inherited by two cells of the endodermal strand of the tailbud embryo although the hybridization signal became weaker (Fig. 4G). Previous *in situ* hybridization experiments of whole-mount ascidian embryos demonstrated that zygotic transcripts first appear in the nuclei, and then transcripts are distributed through the entire cytoplasm (e.g., Yasuo and Satoh, 1993; Satou *et al.*, 1995). The *pem-3* signals were not detected in the nucleus of B7.6 blastomeres or its descendants throughout embryogenesis, suggesting that the signals in the two endodermal strand cells of the tailbud embryo are exclusively maternal.

At the neural-plate stage, strong signals were detected in the presumptive mesenchyme cells, most of the posterior cells (with unknown properties), and part of the anterior a-line epidermal cells (Fig. 4F). In addition, weak signals were detected in all cells forming the neural plate (Fig. 4F). This expression is thought to be zygotic, because signals were present at locations where maternal mRNA had been absent in the early embryos. At the tailbud stage, transcripts were detected in the mesenchyme (Fig. 4G).

To determine the distribution pattern of the PEM-3 protein, a polyclonal antibody against *E. coli*-expressed PEM-3 was prepared. As shown in Fig. 5A, Western blotting indicated that most of the proteins from the maternal transcripts were produced after fertilization, although a small amount of maternal PEM-3 protein was detected. From the 8-cell stage through the gastrula stage, the amount of protein seemed constant. After that, the amount of protein seemed to be decreased. However, at the tailbud stage, the amount of protein slightly increased again. This increase may be due to the zygotic transcript.

Although the eggs were barely stained (data not shown), the whole-mount immunostaining showed the localization of the PEM-3 protein at the posterior side of the 8-cell embryo (Figs. 5B and 5C), the 16-cell embryo (data not shown), and the 32-cell embryo (Figs. 5D and 5E). The distribution domains were the posterior region of the blastomeres where the mRNA was detected, that is, B4.1 at 8-cell embryos, B5.2 at 16-cell embryos, and B6.4 at 32-cell embryos. No signal above background was detected in regions other than the posterior region. After that, the product from the maternal message became barely detectable. At the neural-plate stage, strong staining was detected in the posterior two rows of six neural-plate cells and in anterior a-line epidermal cells, and weak staining was detected in the presumptive mesenchyme region (Figs. 5F–5I). While the mRNA was detected in the entire region of the neural plate (Fig. 4F), PEM-3 was detected only in cells of the posterior two rows of the neural plate. Although the amount of mRNA in mesenchyme cells seems to be more abundant than that in the neural-plate cells (Fig. 4F), the amount of protein in the mesenchyme appeared to be less. These observations suggest that the translational regulation governs the pattern of protein distribution. At the tailbud stage, distinct signal was detected in the anterior epidermis, and faint signals were detected in the mesenchyme and brain region (Figs. 5J and 5K).

Experiments to Deduce pem-3 Functions

The possible RNA-binding ability of PEM-3 and the characteristic expression pattern of the *pem-3* mRNA and its protein indicate some role for PEM-3 in embryogenesis. At first, to explore its putative function, synthetic capped *pem-3* mRNA was injected into ascidian eggs. Three independent series of eggs (total of 58) were injected and, of these, 55 eggs developed into larvae with normal morphology and 3 developed into larvae with a slightly abnormal tail. Synthetic *lacZ* mRNA was also injected as a negative control, and 48 of 52 injected eggs developed into larvae with normal morphology and 4 developed into larvae with a slightly abnormal tail (data not shown). Therefore, it was concluded that *pem-3* overexpression by this method did not show any effects on ascidian larval development.

In ascidians, zygotic mRNAs have been successfully destroyed with antisense oligodeoxynucleotides (ODNs) (Swalla and Jeffery, 1996a; Olson and Jeffery, 1997). Therefore, three sets of antisense ODNs and their control scrambled ODNs (see Materials and Methods) were synthesized to explore the function of zygotic PEM-3. Antisense-1 (AS1) and scramble-1 (SC1) correspond to nucleotide positions 56–35 of the *pem-3* cDNA, AS2 and SC2 to positions 138–121, and AS3 and SC3 to positions 413–394. As another control for AS3, sense ODN-3 (SN3) was also synthesized.

The treatment of fertilized eggs with 2 or 3 μM AS1, SC1, AS2, or SC2 did not show any effects on embryogenesis; the fertilized eggs developed into normal tadpoles. This was confirmed on 50 eggs in each experiment. However, when fertilized eggs were treated with 4 or 5 μM AS1, SC1, AS2, or SC2 they cleaved abnormally, resulting in the formation of cell masses (data not shown).

In contrast to AS1 and AS2, AS3 induced some deficiency in larval development when fertilized eggs were treated with a 2 μM concentration. The results of eggs treated with 2 μM AS3, SC3, or SN3 are summarized in Table 1 and Fig. 6. The experiment was repeated several times using different batches of embryos. The treatment of embryos with the scramble ODN (SC3) or the sense control ODN (SN3) did not have any effects; 173 of the 179 SC3-treated eggs and all of the 91 SN3-treated eggs developed normal larvae (Fig. 6A). However, when fertilized eggs were treated with 2 μM AS3, they developed into abnormal larvae (Figs. 6A′ and 6A″). Cleavage and gastrulation of the treated eggs occurred normally, but the formation of tailbud embryos became irregular, resulting in a larva with shorter tail, as shown in

TABLE 1
Effect of Treatment of Eggs with Antisense Oligonucleotides on the Development of Sensory Pigment Cells

ODN	Number of batches	Number of embryos scored	Number of pigment cells		
			2	1	0
AS3	7	192	6 (3%)	52 (27%)	134 (70%)
SC3	7	179	173 (97%)	5 (2%)	1 (1%)
SN3	4	91	91 (100%)	0 (0%)	0 (0%)

Note. ODN, oligodeoxynucleotide; AS, antisense ODN; SC, scramble ODN, SN, sense ODN.

Figs. 6A′ and 6A″. These larvae were bent dorsally, because their tail elongated to the side where the brain was seen. These abnormal larvae hatched from the chorion slightly later (2–5 h) than did the normal larvae. The AS3-treated larvae showed deficiency of the brain (Figs. 6A′ and 6A″). Almost none of the larvae developed two sensory pigment cells (Table 1 and Fig. 6A′). About 70% of them lacked both pigment cells (Table 1 and Fig. 6A′), and 27% of the embryos developed one pigment cell (Table 1 and Fig. 6A″). This suggests that an antisense ODN inhibits the normal development of the brain. The differentiation of muscle cells was confirmed using the antibody Mu-2, which recognizes muscle-specific myosin heavy chain (Figs. 6B and 6B′; Nishikata *et al.*, 1987; Makabe and Satoh, 1989). Endoderm differentiation was ascertained by the histochemical detection of alkaline phosphatase (Figs. 6C and 6C′). As mentioned earlier, the AS3-treated larvae hatched, suggesting a functional differentiation of epidermis. Sections of a control larva and an AS3-treated larva suggested the differentiation of epidermis, endoderm, and mesenchyme (Figs. 6D and 6D′), while the brain vesicle that was seen in the control embryo (Fig. 6D) was not seen in the AS3-treated embryo (Fig. 6D′).

The specificity of this treatment was further confirmed by the *in situ* hybridization of the AS3-ODN-treated tailbud embryos using *pem-3* antisense probe. The strong signal detected in the mesenchyme cells of control embryos (Fig. 6E) was lacking in those of most of the AS3-treated embryos (Fig. 6E′), although the trunk region of AS3-treated embryos seemed to be stained above background. However, the maternal transcript was detected in the tail region of both embryos (Figs. 6E and 6E′). To ascertain that other mRNAs were transcribed, *in situ* hybridization using the probe that detects the central nervous system and notochord (Chiba *et al.*, unpublished results) was performed. In the AS3-treated embryos, the signal was normally detected in the central nervous system (data not shown). These results indicate that AS3 treatment destroyed the zygotic *pem-3* message specifically and, therefore, the phenotype appeared to be due to the lack of zygotic message and its product. Western blotting of the ODN-treated embryos of the tailbud stage was also performed (Fig. 6F). The amount of PEM-3 protein in the AS3-treated embryos was calculated as 70% of that of the SC3- or SN3-treated embryos. These results strongly suggest that AS3 treatment specifically destroyed the zygotic *pem-3* message and its product, although PEM-3 protein was detected at a certain level even in the AS3-treated embryos (see Discussion).

In addition, to examine the results further, embryos were treated with ODNs after gastrulation. Abnormal larvae with a similar phenotype were obtained by AS3 treatment (data not shown). Therefore, it may be concluded that the development of larvae with a brain deficiency was due to the inhibition of the function of PEM-3 protein that was translated from the zygotically transcribed mRNA.

DISCUSSION

Isolation of cDNA Clones for Maternally Expressed Genes with Localized mRNA in Ascidian Eggs

In the previous study (Satou and Satoh, 1997) and present study, we attempted the isolation and characterization of maternally expressed genes with localized mRNA in the ascidian *Ci. savignyi* by a method different from that used for the isolation of *pem* (Yoshida *et al.*, 1996). We constructed a cDNA library of fertilized egg mRNAs subtracted with gastrula mRNAs and examined about 100 clones that were randomly picked from the library. Of these 5 independent clones (*pem-2*, *pem-3*, *pem-4*, *pem-5*, and *pem-6*), which do not include *pem* cDNA itself, showed localization of the corresponding mRNAs. All of these mRNAs including *pem* showed localization in the posterior-vegetal cytoplasm of eggs and early embryos. mRNAs with a similar localization pattern were also identified in a cDNA project of *Halocynthia roretzi* maternal messages (Sasakura *et al.*, 1998a,b). Swalla and Jeffery (1995, 1996b) also isolated cDNA clones for maternally localized RNA from *Styela clava; yellow crescent* (*YC*) RNA and *PCNA* mRNA are enriched in the myoplasm and the ectoplasm, respectively. In addition, we performed a further screening of the library and isolated several cDNA clones for mRNA that show another localization pattern (Imai *et al.*, 1999). Since, as mentioned earlier, there is convincing evidence of the localization of the determinants responsible for the differentiation of muscle (Nishida, 1992; Marikawa *et al.*, 1994), epidermis (Nishida, 1994a), and endoderm (Nishida, 1993), factors for the establishment of the embryonic anteropos-

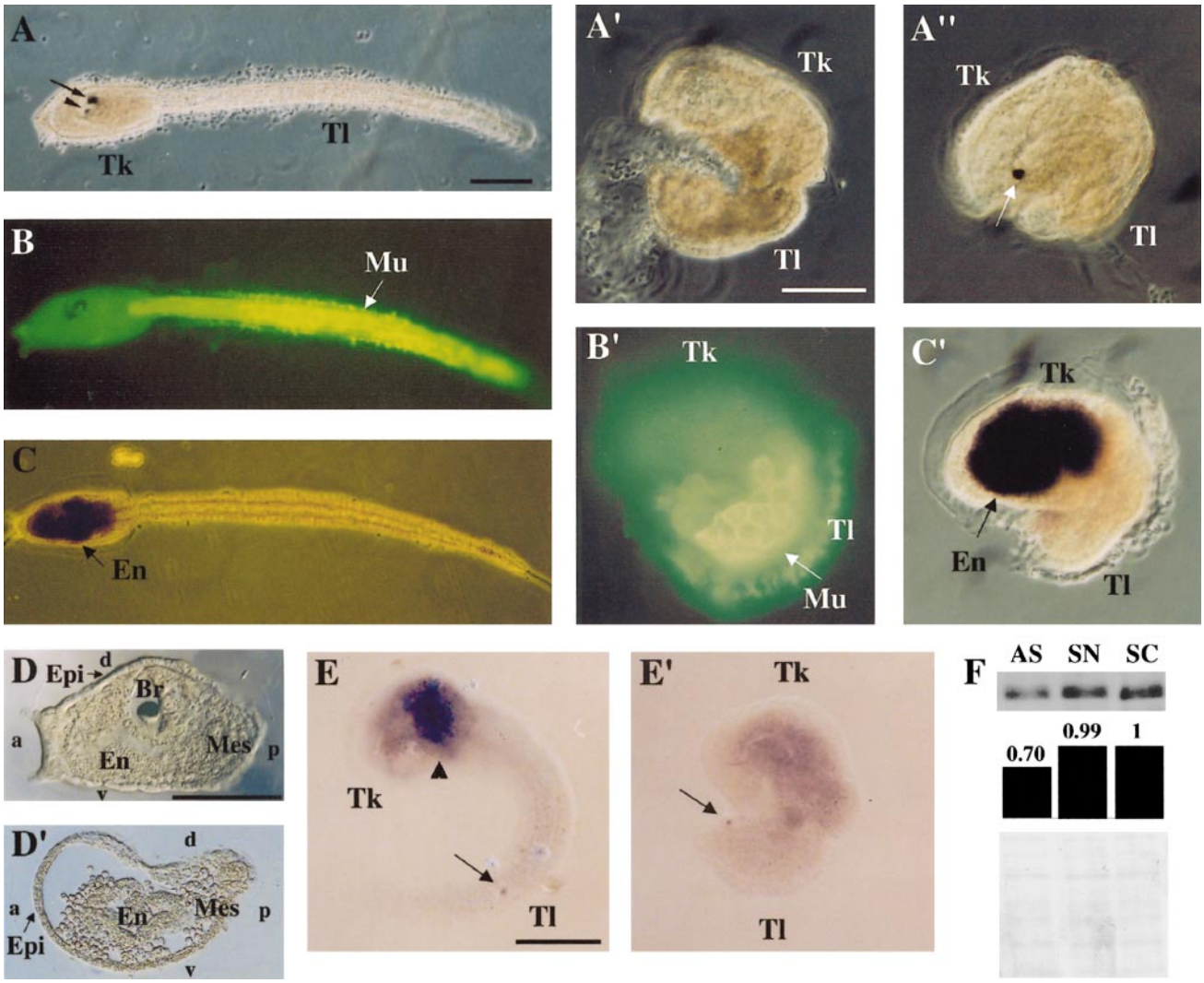

FIG. 6. Effects of *pem-3* antisense oligonucleotide on *Ciona* development. Antisense and control ODNs were penetrated into fertilized eggs, and treated eggs were allowed to develop until the time of hatching of control larvae. (A) An arrowhead shows the otolith, and an arrow shows the ocellus. Tk, trunk; Tl, tail. (A′) An experimental larva of the same age as that in (A) derived from an egg treated with *pem-3* AS3 antisense ODN showing the development of an abnormal larva without the adhesive organ and disturbance of sensory pigment cells. (A″) An experimental larva of the same age as that in (A) derived from an egg treated with *pem-3* AS3 antisense ODN showing the development of an abnormal larva with a sensory pigment cell (arrow). (B, B′) Mu-2 (anti-myosin heavy chain) antibody staining a control larva (B) and an AS3-treated larva (B′), showing the differentiation of muscle cells (Mu). (C, C′) Histochemical staining of alkaline phosphatase in a control larva (C) and an AS3-treated larva (C′), showing the differentiation of endoderm (En). (D, D′) Section of trunk region of a control larva (D) and an AS3-treated larva (D′). Differentiation of epidermis (Epi), endoderm (En), and mesenchyme (Mes) was confirmed, while brain (Br) was not confirmed in the AS3-treated larva. a, anterior; p, posterior; d, dorsal; v, ventral. (E, E′) *In situ* hybridization pattern of tailbud embryos using *pem-3* antisense probe. (E) An SC3 ODN-treated embryo and (E′) an AS3 ODN-treated embryo. While maternal transcript was detected in both embryos (arrows), zygotic transcript was lost in the AS3-treated embryo (E′). (F) Amount of PEM-3 protein in AS3 (AS), SC3 (SC), and SN3 (SN) ODN-treated tailbud embryos. (Top) Western blotting using anti-PEM-3 antibody. (Middle) Relative abundance of protein calculated with an image analyzer. (Bottom) Amide black staining of membrane after signal detection, showing that the same amount of protein was loaded onto each lane. The amount of PEM-3 protein was decreased in the AS3-treated embryos. Scale bars represent 100 μm.

terior axis (Nishida, 1994b), and those for the initiation of gastrulation (Jeffery, 1990; Nishida, 1996), investigations including our studies for localized maternal mRNA will give us new insights into the molecules responsible for ascidian early embryogenesis.

The pattern of localization and segregation of *pem*,

pem-2, pem-3, pem-4, pem-5 and *pem-6* mRNAs and PEM-3 protein observed in the eggs and embryos up to the 8-cell stage closely resemble those of so-called myoplasm. The myoplasm of ascidian eggs contains muscle determinants (Nishida, 1992; Marikawa *et al.,* 1995), factors for the anteroposterior axis formation (Nishida, 1994b), and factors for the initiation of gastrulation (Jeffery, 1990; Nishida, 1996). The myoplasm is a unique cytoskeletal domain (reviewed by Jeffery and Swalla, 1990; Satoh, 1994), which consists of several cytoskeletal proteins (Nishikata *et al.,* 1987; Swalla *et al.* 1991; Marikawa, 1995). However, as shown in the previous study (Yoshida *et al.,* 1996; Satou and Satoh, 1997) and present study, after the 8-cell stage, the localized mRNAs are not segregated with the myoplasm but rather are restricted to the posterior end of the embryo. This suggests the presence of a certain anchoring mechanism in the posterior-most region of the embryo. It has been reported that the 3′ UTRs of mRNAs often contain the information for their localization (reviewed by Ding and Lipshitz, 1993). In the previous study (Satou and Satoh, 1997), we reported that *pem, pem-2, pem-4, pem-5,* and *pem-6* share a six-base motif, "UUAUUU," in their 3′ UTR regions. The *pem-3* cDNA sequence also contains this motif, which is repeated five times in the 5′ and 3′ UTR regions (Fig. 1). One of the key research subjects for future studies is to determine whether this motif is truly important for the localization of the RNAs.

pem-3 Encodes an RNA-Binding Protein

The predicted amino acid sequence of PEM-3 demonstrated that it contains two KH domains. The KH domain was first described in the hnRNP K protein, a pre-mRNA-binding protein (Siomi *et al.,* 1993). The KH domain has now been found in several proteins that are known to interact with RNA, and there is evidence that this motif constitutes a single-stranded RNA-binding domain (Liu and Hanna, 1995; Urlaub *et al.,* 1995). Mutations in several KH domain-containing proteins lead to developmental defects. These include the human fragile-X and mental retardation protein FMR1 (Ashley *et al.,* 1993; Gibson *et al.,* 1993), the *Drosophila* protein BICAUDAL-C (Mahone *et al.,* 1995), the *Ca. elegans* protein GLD-1 (Jones and Schedl, 1995), and MEX-3 (Draper *et al.,* 1996). In addition to the KH domain, PEM-3 also contains the consensus sequence of the RING finger or C3HC4 zinc finger motif (Freemont *et al.,* 1991). Recent studies suggest that the RING finger motif is associated with protein–protein interactions (Rothe *et al.,* 1994; Borden *et al.,* 1995; Saurin *et al.,* 1996). Therefore, it is thought that PEM-3 is an RNA-binding protein with the ability to interact with other proteins, and this may be the first report of a cDNA clone encoding protein containing KH domains and a RING finger.

As mentioned above, the myoplasm is a unique cytoskeletal domain, which anchors several developmentally important maternal factors. In addition, maternal mRNA is a component of muscle determinants (Marikawa *et al.,* 1995). Therefore, it has long been believed that the myoplasm contains RNA-binding proteins. Actually, PEM-3 is localized in the posterior portion of the myoplasm. The results of the present study therefore provide the first evidence of a localized RNA-binding protein in ascidian eggs and early embryos, although its function should be determined in future studies.

Localized RNAs often generate protein gradients, such as Bicoid in *Drosophila* embryo (Driever and Nüsslein-Volhard, 1988). However, the PEM-3 protein did not show a graded expression pattern. Instead, PEM-3 expression was severely restricted to a narrow area around the region where *pem-3* mRNA was expressed. This expression pattern may be important for its function as an RNA-binding protein.

A Developmental Role of PEM-3 in Later Embryogenesis

Two types of experiments were performed to examine PEM-3 function. The overexpression of PEM-3 produced by the microinjection of synthesized *pem-3* mRNA did not affect embryogenesis. The inhibition of the possible function of PEM-3 by the treatment of embryos with antisense oligonucleotides resulted in the development of larvae with a brain deficiency. In ascidians, it was reported that antisense ODNs exert a specific inhibition on embryogenesis, although the antisense ODNs are applicable to zygotically expressed messages but not maternally expressed messages (Swalla and Jeffery, 1996; Olsen and Jeffery, 1997). I examined three types of antisense oligonucleotides as well as their controls. An inhibitory effect was observed only when embryos were treated with the AS3 antisense ODN. As shown in Fig. 6E′, the inhibition was restricted to zygotic *pem-3* transcript; maternal *pem-3* mRNA was not inhibited. A certain level of PEM-3 protein was detected even in the AS3-treated embryos (Fig. 6F). There are two possible reasons for this result. One reason is the presence of PEM-3 protein made from maternal mRNA, although it was not detected in whole-mount immunostaining of tailbud embryos. The other possibility is that the effect of AS3 treatment is not necessarily uniform. The pigment cells of the resulting larvae were not necessarily absent; a significant portion of the embryos had one pigment cell (27%, Table 1). In these embryos, the function of the PEM-3 protein may not have been completely blocked.

The AS3-treated larvae did not form pigment cells, and their tail regions bent dorsally. In the ascidian larvae, two pigment cells (otolith and ocellus) are major components of the brain vesicle. A dorsally bending phenotype is often observed when the neural tube formation fails. The major zygotic expression domains of PEM-3 are the posterior two rows of neural-plate cells; these cells are identified as a9.33, a9.34, a9.37, a9.38, a9.49, and a9.50. The developmental fates of these cells are brain, pigment cells, and primordial pharynx of the larva. Mesenchyme and anterior epidermis also expressed PEM-3. In ascidians, there is no report suggesting that brain formation requires mesenchyme or

anterior epidermis, although it was revealed that the formation requires induction from A-line presumptive endodermal and notochord cells (Reverberi *et al.*, 1960) or spinal cord (Nishida, 1991). Therefore, it is likely that the deficiency of the brain formation is the result of the inhibition of the PEM-3 function in the neural-plate cells.

Several RNA-binding proteins are known to be involved in the development of the chordate CNS. Musashi and Hu are RNA-binding proteins that are expressed in the CNS stem cells and postmitotic CNS cells, respectively, although the RNA-binding domains of these proteins are not KH domains (Marusich *et al.*, 1994; Sakakibara *et al.*, 1996). The *quaking* gene encodes an RNA-binding protein with a KH domain (Ebersole *et al.*, 1996). An analysis of *quaking* mutant mice showed that the *quaking* gene is essential for nervous system myelination and survival of the early embryo, although it has not yet been revealed how the *quaking* gene product works through its RNA-binding activity (Ebersole *et al.*, 1996). PEM-3 is also a putative RNA-binding protein with two KH domains. Because PEM-3 does not have a significant similarity to the proteins described above, it may belong to another class of RNA-binding proteins with the formation and function of neural cells.

Similarity between PEM-3 and MEX-3

The KH domains of PEM-3 show an extensive similarity with those of *Ca. elegans* MEX-3, suggesting that *pem-3* is a candidate homologue of *mex* (*muscle-excess*)*-3* (Fig. 3A). In addition to the sequence similarity, both genes are expressed maternally, and the maternal mRNAs and proteins are localized in early embryos.

Most of the *mex-3* mRNA and protein are distributed in AB and its descendent blastomeres, although both the mRNA and the protein disappear by the 4-cell stage. MEX-3 protein determines the blastomere identity by repressing *pal-1* translation in the AB lineage through the *pal-1* 3′ UTR (Draper *et al.*, 1996). The region with *pem-3* expression is different from that of *mex-3;* maternal *pem-3* mRNA and protein are localized in the posterior pole of the ascidian embryos, while *mex-3* mRNA and protein are expressed in anterior blastomeres of nematode embryos. However, it is possible that maternal PEM-3 is also required for blastomere identification in ascidian embryos. In fact, it was indicated that *pem* with the same expression pattern as *pem-3* has a function involved in the anterior–posterior axis (Yoshida *et al.*, 1996, 1998). If maternal PEM-3 had a similar function, it would be the first example of a conserved function and mechanism of maternal RNAs that regulate cell identity.

There is an alternative possibility. MEX-3 protein is also a component of P-granules (Draper *et al.*, 1996), which are retained in germ cells throughout the life cycle of *Ca. elegans*. Although, in ascidian, it has not yet been demonstrated which cells are the primordial germ cells, several experiments strongly suggest that B7.6 blastomeres are the primordial germ cells (T. Hirano *et al.*, personal communication; K. Takamura *et al.*, personal communication), and B7.6 blastomeres contain maternal *pem-3* mRNA and protein. Therefore, the expression of maternal *pem-3* in B7.6 cells may correspond to the expression of MEX-3 in P-granules. The functions of both maternal PEM-3 in B7.6 blastomeres and MEX-3 in P-granules have not yet been clarified. It is important to determine in future studies what the function of maternal PEM-3 is and whether PEM-3 is an orthologue of MEX-3.

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